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(57) Abstract

Polynucleotides encoding a carboxylesterase enzyme and polypeptides encoded by the polynucleotides which are capable of metabolizing a chemotherapeutic prodrug and inactive metabolites thereof to active drug are provided. Compositions and methods for sensitizing tumor cells to a prodrug chemotherapeutic agent and inhibiting tumor growth with this enzyme are also provided. In addition, screening assay for identification of drugs activated by this enzyme are described.

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COMPOSITIONS AND METHODS FOR SENSITIZING AND INHIBITING GROWTH OF HUMAN TUMOR CELLS

Introduction

This invention was supported in part by funds from the U.S. Government NIH Grant Nos. CA-66124 and CA-63512 and the U.S. Government may therefore have certain rights in the invention.

Field of the Invention

This invention relates to novel polynucleotides identified and sequenced which encode a carboxylesterase enzyme, polypeptides encoded by these polynucleotides and vectors and host cells comprising these vectors which express the enzyme. This enzyme is capable of metabolizing chemotherapeutic prodrugs and inactive metabolites into active drug. The instant invention thus relates to compositions comprising these polynucleotides and methods for sensitizing selected tumor cells to a chemotherapeutic prodrug by transfecting the tumor cells with a polynucleotide placed under the control of a disease-specific responsive promoter. Sensitized tumor cells can then be contacted with a chemotherapeutic prodrug to inhibit tumor cell growth. Compositions of the present invention can also be used in combination with chemotherapeutic prodrugs to purge bone marrow of tumor cells. The invention further includes novel drug screening assays for identifying chemotherapeutic prodrugs that are activated by this enzyme.

Background of the Invention

Cancer is a disease resulting from multiple changes at the genomic level. These changes ultimately lead to the

malfunction of cell cycle machinery and finally to autonomous cell proliferation. Neoplastic transformation involves four types of genes: oncogenes, tumor-suppressor genes, mutator genes, and apoptotic genes. Different types of cancer can involve alteration of any one or any combination of these genes.

Proto-oncogenes of the *myc* family are overexpressed in many different types of human tumors including tumors of the breast, colon, cervix, head and neck, and brain. Many solid tumors amplify or overexpress c-*myc*, with up to a 50-fold increase in c-*myc* RNA in tumor cells relative to normal cells having been reported (Yamada, H. et al. 1986. *Jpn. J. Cancer Res.* 77:370-375). For example, three of the six most common solid tumors, including up to 100% of colon adenocarcinomas, 57% of breast cancers, and 35% of cervical cancers, demonstrate increased levels of c-*myc* protein. Enforced expression of c-*myc* in nontumorigenic cells causes immortalization but not transformation; however, elevated levels of c-*myc* protein are rare in benign cancers and normal differentiated tissue. While solid tumors can oftentimes be removed surgically, overexpression of c-*myc* has been linked with amplification of the c-*myc* gene and correlated with poor prognosis and an increased risk of relapse (Nagai, M.A. et al. 1992. *Dis. Colon Rectum* 35:444-451; Orian, J.M. et al. 1992. *Br. J. Cancer* 66:106-112; Riou, G. et al. 1987. *Lancet* 2:761-763; Field, J.K. et al. 1989. *Oncogene* 4:1463-1468).

Another member of the *myc* oncogene family, N-*myc*, has been linked with development of neuroblastomas in young children. Overexpression of this member of the *myc* family of proto-oncogenes has also been correlated with advanced stages of disease and poor prognosis (Brodeur, G.M. et al. 1997. *J. Ped. Hematol. Oncol.* 19:93-101). Primary tumors for this specific condition usually arise in the abdomen and as many as 70% of patients have bone marrow metastases at diagnosis

(Matthay, K.E. 1997. *Oncology* 11:1857-1875). Treatment of children with Stage 4 disease using surgery, chemotherapy, and purged autologous or allogeneic marrow transplant produces a progression-free survival rate of 25 to 49% in patients four years post transplant (Matthay, K.K. et al. 1994. *J. Clin. Oncol.* 12:2382-2389). Most relapses after autotransplant occur at sites of bulk disease and/or previously involved sites. Estimates of the rate of local recurrence vary depending upon the study. However, recurrence of tumor at an original site has been estimated to occur in approximately 25% of high risk neuroblastoma patients.

Further, definitive evidence from gene marking studies indicates that autologous marrow, free of malignant cells by standard clinical and morphologic criteria, contributes to relapse at both medullary and extramedullary sites (Rill, D.R. et al. 1994. *Blood* 84:380-383). In a recent pilot clinical study, bone marrow involvement at diagnosis correlated with specific relapse at that site in children receiving autologous purged marrow (Matthay, K.K. et al. 1993. *J. Clin. Oncol.* 11:2226-2233). Accordingly, improvements in surgery, detection of tumor margins, development of new anticancer drugs or application of novel therapies are required to prevent local tumor regrowth. In particular, more effective treatment strategies are needed for elimination of "minimal residual disease" or "MRD" which results from the presence of a small number of tumor cells at the site of disease after treatments such as tumor resection or purging bone marrow of tumor cells.

CPT-11 (irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin) is a prodrug currently under investigation for the treatment of cancer that is converted to the active drug known as SN-38 (7-ethyl-10-hydroxy-camptothecin) (Tsuji, T. et al. 1991. *J. Pharmacobiol. Dynamics* 14:341-349; Satoh, T. et al. 1994. *Biol. Pharm. Bull.* 17:662-664). SN-38 is a potent inhibitor of topoisomerase I

(Tanizawa, A. et al. 1994. *J. Natl. Cancer Inst.* 86:836-842; Kawato, Y. et al. 1991. *Cancer Res.* 51:4187-4194), an enzyme whose inhibition in cells can result in DNA damage and induction of apoptosis (Hsiang, Y.-H. et al. 1989. *Cancer Res.* 49:5077-5082). The specific enzyme responsible for activation *in vivo* of CPT-11 has not been identified, although serum or liver homogenates from several mammalian species have been shown to contain activities that convert CPT-11 to SN-38 (Tsuji, T. et al. 1991. *J. Pharmacobiol. Dynamics* 14:341-349; Senter, P.D. et al. 1996. *Cancer Res.* 56:1471-1474; Satoh, T. et al. 1994. *Biol. Pharm. Bull.* 17:662-664). Uniformly, these activities have characteristics of carboxylesterase (CE) enzymes (Tsuji, T. et al. 1991. *J. Pharmacobiol. Dynamics* 14:341-349; Senter, P.D. et al. 1996. *Cancer Res.* 56:1471-1474; Satoh, T. et al. 1994. *Biol. Pharm. Bull.* 17:662-664). In fact, SN-38 can be detected in the plasma of animals and humans minutes after the administration of CPT-11 (Stewart, C.F. et al. 1997. *Cancer Chemother. Pharmacol.* 40:259-265; Kaneda, N. et al. 1990. *Cancer Res.* 50:1715-1720; Rowinsky, E.K. et al. 1994. *Cancer Res.* 54:427-436), suggesting that a CE enzyme present in either serum or tissues can convert the camptothecin analog to its active metabolite.

CEs are ubiquitous serine esterase enzymes that are thought to be involved in the detoxification of a variety of xenobiotics. CEs are primarily present in liver and serum, however, the physiological role of this class of enzymes has yet to be identified. A recent biochemical analysis of 13 CEs compared their ability to metabolize CPT-11 to SN-38. While the efficiency of conversion varied between enzymes, those isolated from rodents were the most efficient (Satoh, T. et al. 1994. *Biol. Pharm. Bull.* 17:662-664). The amino acid sequence of a rabbit liver CE has been disclosed (Korza, G. and J. Ozols. 1988. *J. Biol. Chem.* 263:3486-3495). In addition, there are currently 13 cDNA sequences encoding CE

in the Genbank and EMBL databases, including a rat serum and rat liver microsomal CE. Interestingly, CEs purified from human tissues demonstrated the least efficient conversion of CPT-11 to SN-38, with less than 5% of the prodrug being converted to active drug (Leinweber, F.J. 1987. *Drug Metab. Rev.* 18:379-439; Rivory, L.P. et al. 1997. *Clin. Cancer Res.* 3:1261-1266).

In addition to metabolism to SN-38, in humans CPT-11 is also metabolized to a compound known as APC (Haaz, M.C. et al. 1998. *Cancer Res.* 58:468-472). APC has little, if any, anti-tumor activity and is not converted to an active metabolite in humans (Rivory, L.P. et al. 1996. *Cancer Res.* 56:3689-3694).

In preclinical studies, CPT-11 administered to immune-deprived mice bearing human tumor xenografts produces complete regression of glioblastomas, rhabdomyosarcomas (RMS), neuroblastomas, and colon adenocarcinomas (Houghton, P.J. et al. 1995. *Cancer Chemother. Pharmacol.* 36:393-403; Houghton, P.J. et al. 1993. *Cancer Res.* 53:2823-2829). However, maintenance of tumor regression in studies with CPT-11 appears to be dependent upon drug scheduling, suggesting that viable tumor cells survive therapy (i.e., minimal residual disease (MRD)). These studies also showed a steep dose-response relationship between dose of drug administered and induction of tumor regression. For example, 20 mg of CPT-11/kg/day given daily for 5 days for two weeks produced complete regressions of Rh18 RMS xenografts, while 10 mg/kg/day given on the same schedule produced only partial tumor regression. Similar effects were seen when mice bearing SJGC3A colon adenocarcinoma xenografts were treated with 40 mg CPT-11/kg compared to a 20 mg/kg dose.

Early clinical trials with CPT-11 indicate that the prodrug also has anti-tumor activity *in vivo* against many different types of solid tumors in humans. However, myelosuppression and secretory diarrhea limit the amount of

drug that can be administered to patients. Accordingly, before this promising anti-cancer agent can be used successfully, these dose-limiting toxicities must be overcome.

The development of new effective treatment strategies for cancer is dependent upon the availability of specific drug screening assays. Specific drug screening assays can involve isolated target tissue models, i.e., isolated heart, ileum, vasculature, or liver from animals such as rabbits, rats, and guinea pigs, wherein the target tissue is removed from the animal and a selected activity of that target tissue is measured both before and after exposure to the candidate drug. An example of a selected activity measured in drug screening assays to identify new cancer agents is the activity of enzymes such as topoisomerase I or II, which are known to modulate cell death. Such assays can also be used to screen for potential prodrugs which are converted to the active metabolite in selected tissues or to identify selected tissues capable of converting prodrug to its active metabolite.

However, any molecular event that is shown to be modified by a novel class of compounds can be developed as a screening assay for selection of the most promising compounds for therapeutic development. In fact, in recent years the idea of modulating cells at the genomic level has been applied to the treatment of diseases such as cancer. Gene therapy for treatment of cancer has been the focus of multiple clinical trials approved by the National Institutes of Health Recombinant DNA Advisory Committee, many of which have demonstrated successful clinical application (Hanania et al. 1995. *Am. Jour. Med.* 99:537-552; Johnson et al. 1995. *J. Am. Acad. Derm.* 32(5):689-707; Barnes et al. 1997. *Obstetrics and Gynecology* 89:145-155; Davis et al. 1996. *Current Opinion in Oncology* 8:499-508; Roth and Cristiano 1997. *J. Natl. Canc. Inst.* 89(1):21-39). To specifically target malignant cells and spare normal tissue, cancer gene therapies must combine selective gene delivery with specific gene expression,

specific gene product activity, and, possibly, specific drug activation. Significant progress has been made in recent years using both viral (retrovirus, adenovirus, adeno-associated virus) and nonviral (liposomes, gene gun, injection) methods to efficiently deliver DNA to tumor sites. Genes can be transfected into cells by physical means such as scrape loading or ballistic penetration, by chemical means such as coprecipitation of DNA with calcium phosphate or liposomal encapsulation; or by electro-physiological means such as electroporation. The most widely used methods, however, involve transduction of genes by means of recombinant viruses, taking advantage of the relative efficiency of viral infection processes. Current methods of gene therapy involve infection of organisms with replication-deficient recombinant viruses containing the desired gene. The replication-deficient viruses most commonly used include retroviruses, adenoviruses, adeno-associated viruses, lentiviruses and herpes viruses. The efficacy of viral-mediated gene transfer can approach 100%, enabling the potential use of these viruses for the transduction of cells *in vivo*.

Adenovirus vector systems in particular have several advantages. These include the fact that non-dividing cells can be transduced; transduced DNA does not integrate into host cell DNA, thereby negating insertional mutagenesis; the design of adenoviral vectors allows up to 7 kb of foreign DNA to be incorporated into the viral genome; very high viral titers can be achieved and stored without loss of infectivity; and appropriate plasmids and packaging cell lines are available for the rapid generation of infectious, replication-deficient virus (Yang, N.S. 1992. *Crit. Rev. Biotechnol.* 12:335-356). The effectiveness of adenoviral-mediated delivery of genes into mammalian cells in culture and in animals has been demonstrated.

To increase the specificity and safety of gene therapy for treatment of cancer, expression of the therapeutic gene

within the target tissue must also be tightly controlled. For tumor treatment, targeted gene expression has been analyzed using tissue-specific promoters such as breast, prostate and melanoma specific promoters and disease-specific responsive promoters such as carcinoembryonic antigen, HER-2/neu, Myc-Max response elements, DF3/MUC. Dachs, D.U. et al. 1997. *Oncol. Res.* 9(6-7):313-25. For example, the utility of herpes simplex virus thymidine kinase (HSV-TK) gene ligated with four repeats of the Myc-Max response element, CACGTG (SEQ ID NO:22), as a gene therapy agent for treatment of lung cancer with ganciclovir was examined in c-, L- or N-myc-overexpressing small cell lung cancer (SCLC) cell lines (Kumagai, T. et al. 1996. *Cancer Res.* 56(2):354-358). Transduction of the HSV-TK gene ligated to this CACGTG (SEQ ID NO:22) core rendered individual clones of all three SCLC lines more sensitive to ganciclovir than parental cells *in vitro*, thus suggesting that a CACGTG-driven HSV-TK gene may be useful for the treatment of SCLC overexpressing any type of *myc* family oncogene. Additional experiments with *c-myc* have focused on the use of the ornithine decarboxylase (ODC) promoter gene. Within the first intron of the ODC gene are two CACGTG "E boxes" that provide binding sites for the *c-myc* protein when bound to its partner protein known as max. Mutation of the E box sequence results in the inability of *c-myc* to transactivate the ODC promoter. Previous reports indicate that reporter constructs containing the ODC promoter fused upstream of the chloramphenicol acetyltransferase gene immediately adjacent to the second exon were activated in cells that overexpress *c-myc* (Bello-Fernandez, C. et al. 1993. *Proc. Natl Acad. Sci. USA* 90:7804-7808). In contrast, transient transfection of promoter constructs in which the E boxes were mutated (CACGTG (SEQ ID NO:22) to CACCTG (SEQ ID NO:25) demonstrate significantly lower reporter gene activity. These data suggest that it is possible to activate

transcription of specific genes under control of the *c-myc* responsive ODC promoter. In the case of *N-myc*, *N-myc* protein is a basic helix-loop-helix (BHLH) protein that can dimerize with proteins of the same class. *N-myc* dimerizes with the BHLH protein max to form a complex that binds to the CACGTG motif present in gene promoters, such as ODC, resulting in transactivation and expression of specific genes containing this sequence (Lutz, W. et al. 1996. *Oncogene* 13:803-812). Studies in a neuroblastoma cell line and tumors have shown that binding of *N-myc* to its consensus DNA binding sequence correlates with *N-myc* expression, data that indicate that the level of *N-myc* in neuroblastoma cells is a determining factor in expression of proteins under control of promoters containing the CACGTG sequence (Raschella, G. et al. 1994. *Cancer Res.* 54:2251-2255). Inhibition of expression of the *c-myc* gene via antisense oligonucleotides as a means for inhibiting tumor growth has also been disclosed (Kawasaki, H. et al. 1996. *Artif. Organs* 20(8):836-48).

In the present invention, polynucleotides encoding a carboxylesterase enzyme or active fragments thereof and polypeptides encoded thereby which are capable of metabolizing the chemotherapeutic prodrug CPT-11 and its inactive metabolite APC to active drug SN-38 are disclosed. Use of this enzyme in combination with APC renders this inactive metabolite a useful chemotherapeutic prodrug. It has also been found that compositions comprising a polynucleotide of the present invention and a disease-specific responsive promoter can be delivered to selected tumor cells to sensitize the tumor cells to the chemotherapeutic prodrug CPT-11, thereby inhibiting tumor cell growth.

Summary of the Invention

An object of the present invention is to provide polynucleotides encoding a carboxylesterase capable of

metabolizing a chemotherapeutic prodrug and inactive metabolites thereof to active drug.

Another object of the present invention it to provide polypeptides encoded by these polynucleotides.

Another object of the present invention is to provide vectors comprising these polynucleotides and host cells containing these vectors which express a carboxylesterase.

Another object of the present invention is to provide a composition comprising a polynucleotide encoding a carboxylesterase and a disease-specific responsive promoter of selected tumor cells or a promoter such as CMV.

Another object of the present invention is to provide a method for sensitizing tumor cells to a chemotherapeutic prodrug which comprises transfecting selected tumor cells with a composition comprising a polynucleotide encoding carboxylesterase and a disease-specific responsive promoter of the selected tumor cells.

Another object of the present invention is to provide a method of inhibiting growth of selected tumor cells which comprises sensitizing selected tumor cells to a chemotherapeutic prodrug metabolized to active drug by a carboxylesterase and administering a chemotherapeutic prodrug.

Another object of the present invention is to provide a method of using APC as a prodrug in the treatment of cancer.

Yet another object of the present invention is to provide drug screening assays for identification of compounds activated by a carboxylesterase.

Brief Description of the Figures

Figure 1 shows the homology of N-terminal amino acid sequences of rabbit liver carboxylesterase (CE) enzyme (SEQ ID NO:1) with other known CEs including rabbit (P12337;SEQ ID NO:2), human (P23141;SEQ ID NO:3), rat (P10959;SEQ ID NO:4), and mouse (P23953;SEQ ID NO:5). The vertical lines indicate the homology of the sequenced CE with the rabbit protein

sequence in the Swissprot database. Underlined residues in the rabbit sequence indicate amino acids conserved among all the CE proteins.

Figure 2 shows the design of the oligonucleotides used for degenerate PCR. The amino acid sequence (SEQ ID NO:6) and the coding sequence (SEQ ID NO:7) of residues 1 through 5 of rabbit CE are depicted along with the corresponding oligonucleotide Rab51 (SEQ ID NO:8) and Rab52 (SEQ ID NO:9). Also depicted are the amino acid sequence (SEQ ID NO:10), the coding sequence (SEQ ID NO:11) and the reverse complement (SEQ ID NO:12) of residues 518 through 524 of rabbit CE, along with oligonucleotide Rab31 (SEQ ID NO:13) and Rab32 (SEQ ID NO:14).

Figure 3 shows the alignment of N-terminal signal sequences of the rabbit liver CE (SEQ ID NO:15) and other known CEs including rat (P10959; SEQ ID NO:16), human (P23141; SEQ ID NO:17), rat (16303; SEQ ID NO:18) and mouse (P23953; SEQ ID NO:19). Residues common to all CEs are underlined and the 18 residue leader sequence is indicated in italics. The Swissprot Accession numbers are indicated in parentheses.

Figure 4 shows the complete coding sequence of the rabbit liver CE (SEQ ID NO:20) and the amino acid sequence encoded thereby (SEQ ID NO:21). The 1698 bp ORF encodes a 62.3 kDa protein. The N-terminal hydrophobic leader sequence is in italics, the 5' and 3' RACE sequences are underlined and the potential active site serine is indicated by an asterisk. The carboxylesterase B-1 and B-2 motifs, at amino acids 208-223 and 114-124 are double underlined.

Figure 5 is a linegraph comparing % cell survival, depicted on the Y-axis, at various concentrations of CPT-11, depicted on the X-axis. Control Cos7 cells (filled squares) are approximately 350-fold more sensitive to CPT-11 than Cos7 cell transfected with CE (filled triangles).

Figure 6 is a linegraph showing the conversion of APC, depicted on the X-axis at nanomolar concentrations, to SN-38, depicted on the Y-axis at nanomolar concentrations, *in vitro* by the activity of rabbit liver CE given at doses of 0 (filled cross), 10 (filled hexagon), 25 (filled triangle), 50 (filled circle) or 100 (filled square) units. Data presented represent the mean response at each dose level.

Figure 7 is a linegraph showing a comparison of the sensitization, depicted as % survival on the Y-axis, of U-373 glioma cells exposed to APC, depicted as log[APC] at concentrations from 10^{-8} to 10^{-5} M on the X-axis, from *in situ* expression of rabbit liver CE (filled squares) and human alveolar macrophage CE (filled circles). Cells were exposed for 2 hours to APC.

Figure 8 provides the chemical structures of CPT-11, APC and SN-38.

Figure 9A, 9B, and 9C are linegraphs showing the responses of mice bearing Rh30 and Rh30pIRES$_{rabbit}$ rhabdosarcoma xenografts to CPT-11 treatment. Each line on each graph shows the growth of an individual tumor. The tumor growth rate is depicted on the Y-axis of each graph in terms of tumor volume and is plotted as a function of time in weeks (X-axis). Figure 9A depicts cells expressing rabbit CE (Rh30pIRES$_{rabbit}$) not treated with CPT-11. Figure 9B depicts cells expressing rabbit CE (Rh30pIRES$_{rabbit}$) and then treated with CPT-11 and shows complete tumor regression, even out to 12 weeks. Figure 9C depicts control cells (Rh30) exposed to CPT-11 and shows initial regression but regrowth.

Figure 10 is a linegraph showing the effects of CPT-11 treatment on U373 glioblastoma xenografts expressing rabbit CE. Mice bearing xenografts were treated with CPT-11 (7.5 mg/kg for 5 days) for three treatment cycles. The tumor growth rate is depicted on the Y-axis in terms of tumor volume and is plotted as a function of time in weeks (X-axis). Open circles depict the tumor volume of untreated U373 xenografts

expressing rabbit CE. Filled triangles depict the response of control xenografts (no rabbit CE) treated with CPT-11. Filled squares depict the response of cells expressing rabbit CE and treated with CPT-11. The data show that tumor regression was seen only in treated cells expressing rabbit CE. Each point represents the mean of 14 tumors in 7 individual mice.

Detailed Description of the Invention

CPT-11 is a promising anti-cancer prodrug, that when given to patients, is converted to its active metabolite SN-38 by a human carboxylesterase. However, the human enzyme is relatively inefficient and less than 5% of the prodrug is metabolized to SN-38 (Rivory, L.P. et al. 1997. *Clin. Cancer Res.* 3:1261-1266). In patients, this prodrug is also metabolized to APC (Haaz, M-C. et al. 1998. *Cancer Res.* 58:468-472). APC has little, if any, active anti-tumor activity and is not converted to an active metabolite in humans (Rivory, L.P. et al. 1996. *Cancer Res.* 56:3689-3694). Accordingly, high concentrations of this prodrug must be administered to achieve effective levels of active drug *in vivo*. However, myelosuppression and secretory diarrhea limit the amount of prodrug that can be administered to patients.

In the present invention, a method of sensitizing tumor cells to reduce the effective dose of a prodrug required to inhibit tumor cell growth is provided which comprises transfecting selected tumor cells with a polynucleotide under the control of a disease-specific responsive promoter such as a *myc* promoter. The present invention exploits the tumor-specific overexpression of oncogenes of the *myc* family to produce selective killing with a chemotherapeutic prodrug.

In accordance with one aspect of the present invention there are provided polynucleotides which encode a carboxylesterase capable of metabolizing a chemotherapeutic prodrug and inactive metabolites thereof to active drug. By

"polynucleotides" it is meant to include any form of DNA or RNA such as cDNA or genomic DNA or mRNA, respectively, encoding this enzyme or an active fragment thereof which are obtained by cloning or produced synthetically by well known chemical techniques. DNA may be double- or single-stranded. Single-stranded DNA may comprise the coding or sense strand or the non-coding or antisense strand. Thus, the term polynucleotide also includes polynucleotides which hybridize under stringent conditions to the above-described polynucleotides. As used herein, the term "stringent conditions" means at least 60% homology at hybridization conditions of 60°C at 2xSSC buffer. In a preferred embodiment, the polynucleotide comprises the cDNA depicted in Figure 4 (SEQ ID NO:20) or a homologous sequence or fragment thereof which encodes a polypeptide having similar activity to that of this rabbit liver CE enzyme. Due to the degeneracy of the genetic code, polynucleotides of the present invention may also comprise other nucleic acid sequences encoding this enzyme and derivatives, variants or active fragments thereof. The present invention also relates to variants of this polynucleotide which may be naturally occurring, i.e., allelic variants, or mutants prepared by well known mutagenesis techniques.

Also provided in the present invention are vectors comprising polynucleotides of the present invention and host cells which are genetically engineered with vectors of the present invention to produce CE or active fragments of this enzyme. Generally, any vector suitable to maintain, propagate or express polynucleotides to produce the enzyme in the host cell may be used for expression in this regard. In accordance with this aspect of the invention the vector may be, for example, a plasmid vector, a single- or double-stranded phage vector, or a single- or double-stranded RNA or DNA viral vector. Such vectors include, but are not limited to, chromosomal, episomal and virus-derived vectors e.g., vectors derived from bacterial plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses such as

baculoviruses, papova viruses, SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids and phagemids. Selection of an appropriate promoter to direct mRNA transcription and construction of expression vectors are well known. In general, however, expression constructs will contain sites for transcription initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating codon at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated. Examples of eukaryotic promoters routinely used in expression vectors include, but are not limited to, the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous Sarcoma Virus(RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter. Vectors comprising the polynucleotides can be introduced into host cells using any number of well known techniques including infection, transduction, transfection, transvection and transformation. The polynucleotides may be introduced into a host alone or with additional polynucleotides encoding, for example, a selectable marker. Host cells for the various expression constructs are well known, and those of skill can routinely select a host cell for expressing the rabbit liver CE enzyme in accordance with this aspect of the present invention. Examples of mammalian expression systems useful in the present invention include, but are not limited to, the C127, 3T3, CHO, HeLa, human kidney 293 and BHK cell lines, and the COS-7 line of monkey kidney fibroblasts. Alternatively, as exemplified herein, rabbit CE can be expressed in Spodoptera frugiperda Sf21 cells via a baculovirus vector (see Example 3).

The present invention also relates to compositions comprising a polynucleotide of the present invention which

have been found to be useful in sensitizing tumor cells to CPT-11 cytotoxicity by combination therapy of the prodrug and a CE enzyme. The present invention thus provides methods for sensitizing tumor cells to a prodrug oncologic agent. In this context, by "sensitizing" it is meant that the effective dose of the prodrug can be reduced when the compositions and methods of the present invention are employed. In a case where the prodrug's therapeutic activity is limited by the occurrence of significant toxicities, or dose-limiting toxicities, sensitization of tumor cells to the prodrug is especially useful.

In one embodiment, selected tumor cells are transfected with the cDNA of the present invention and expressed via a well known promoter such as the CMV promoter or, more preferably, via a disease-specific responsive promoter which specifically targets the selected tumor cells. Targeted gene expression in tumor cells has been achieved using disease-specific responsive promoters such as carcinoembryonic antigen, HER-2/neu, Myc-Max response elements, and DF3/MUC. Thus, a composition comprising the cDNA rabbit liver CE and a disease-specific responsive promoter such as these can be used to transfect and sensitize tumor cells containing the disease-specific responsive promoter. Accordingly, the present invention provides a means for exploiting tumor-specific expression associated with a disease-specific responsive promoter to provide for selective therapy of tumors.

Since *myc* expression is deregulated in a wide variety of human tumors, *myc* is an attractive target for chemotherapeutics. No known drug specifically interacts with either the *c-myc* or *N-myc* protein. However, cells overexpressing a *myc* oncogene can be targeted with compositions of the present invention comprising a polynucleotide of the present invention under the control of a *myc* specific promoter. Thus, using the present invention the tumor-specific overexpression of *c-myc* and N-*myc* can be

exploited to produce selective killing with a chemotherapeutic agent. Specifically, transcription of genes under the control of the promoter containing the CACGTG (SEQ ID NO:22) binding sequence of either N-*myc* or *c-myc* are upregulated in cells overexpressing these *myc* genes, producing tumor cell-specific expression of the polynucleotide encoding the CE that is capable of activating the chemotherapeutic prodrug CPT-11.

The ability of a promoter to regulate gene expression was confirmed in cell lines overexpressing *c-myc*, SJ-G2 and NCI-H82 cells (which overexpress *c-myc*) and Rh28 cells (which have no detectable levels of *c-myc* protein). In these experiments, cells were transiently transfected with a plasmid containing the ODC promoter controlling expression of a reporter gene for chloramphenicol acetyltransferase. A mutated ODC promoter in which *c-myc* transactivation domains have been inactivated by point mutations was used as a control. A 4 to 5 fold increase in reporter activity was observed in SJ-G2 cells and NCI-H82 cells, respectively, following transfection with the plasmid containing native ODC promoter as compared to the mutant promoter sequence. No significant increase in promoter activity was observed in Rh28 cells. These results are consistent with *c-myc*-mediated activation of transcription by binding to the cognate sequence within the ODC promoter. In addition, the levels of activation were similar to that seen with reporter constructs when enforced co-expression of *c-myc* occurs during transfection of CV-1 and NIH-3T3 cells.

The cDNA depicted in Figure 4 (SEQ ID NO:20) was isolated by synthesizing degenerate oligonucleotides from amino acid residues 1-5 (SEQ ID NO:6) and 518-524 (SEQ ID NO:10) of a published rabbit CE protein sequence (Korza, G. and J. Ozols. 1988. *J. Biol. Chem.* 263:3486-3495). The oligonucleotides constructed are shown in Figure 2. To amplify the rabbit cDNA by PCR, cDNA was prepared from rabbit liver poly A+ mRNA and multiple samples were prepared that

contained the combination of oligonucleotide primers. Using PCR techniques, a single product was obtained from one set of reactions that upon DNA sequencing was shown to encode the rabbit CE.

Since this represented a partial cDNA, both 5' and 3' RACE were used to amplify the entire coding sequence. Unique primers were designed from the partial DNA sequence. These oligonucleotides were used in combination with the AP1 primer to amplify sequences prepared from Marathon adapted rabbit liver cDNA. Touchdown PCR (Don, R.H. et al. 1991. *Nucleic Acids Res.* 19:4008) was performed in accordance with the Marathon cDNA amplification protocol.

The complete sequence of the cDNA (SEQ ID NO:20) and the derived amino acid sequence (SEQ ID NO:21) of a rabbit liver CE are shown in Figure 4. Northern analysis of the poly A+ mRNA from the rabbit liver with a [^{32}P]-labeled cDNA confirmed the presence of a single transcript of approximately 1.84 knt. No cross reaction was observed with any other mRNA, consistent with this cDNA representing a unique RNA species.

Further, comparison of the amino acid sequence of the polypeptide encoded by the cDNA of the present invention with the published amino acid sequence for rabbit CE (Swissprot Accession Number P12337; Korza, G. and J. Ozols. 1988. *J. Biol. Chem.* 263:3486-3495) showed three mismatches. In addition, the polypeptide encoded by the cDNA of the present invention contains an 8 amino acid insert and an 18 amino acid leader sequence at the N-terminus which the published sequence does not contain. Accordingly, another aspect of the present invention relates to novel polypeptides encoded by polynucleotides of the present invention. By "polypeptide" it is meant to include the amino acid sequence of SEQ ID NO: 21 depicted in Figure 4 and fragments, derivatives and analogs which retain essentially the same biological activity and/or function as this rabbit liver CE.

The rabbit cDNA was expressed in bacteria. The 1.7 kb cDNA was ligated into pET32b and transformed into *E. coli*

L21(DE3). Two clones were isolated containing the rabbit cDNA either in the correct (pETRABFL) or incorrect (pETLFBAR) orientation with respect to the T7 promoter. Following induction of expression in liquid culture with IPTG, cell extracts were analyzed by SDS-PAGE and Western blotting. A 75 kDa protein resulted from the fusion of the rabbit CE with the thioredoxin protein in pETRABFL. Western analysis with the rat liver microsomal CE antibody and horseradish peroxidase (HRP)-conjugated protein S confirmed that the 75 kDa protein encoded by pETRABFL contained the rabbit CE. Since other CEs are located in the ER and the primary sequence of the rabbit enzyme contains similar characteristic leader and anchor sequences (Satoh, T. and M. Hosokawa. 1995. *Toxicol. Lett.* 82/83:439-445), it is likely that the compartmentalization of the CE to the ER is required for enzymatic activity. Indeed, overexpression of the human alveolar macrophage CE in *E. coli* failed to generate CE activity, however transfection of mammalian cells with the same cDNA yielded significant conversion of o-NPA by whole cell extracts. In addition, the rabbit CE demonstrated greater than 85% homology with human alveolar macrophage CE yet the latter enzyme failed to convert CPT-11 to SN-38 in mammalian cells. This indicates that while CEs may have a broad range of substrate specificities, the efficiency with which similar enzymes within different species can utilize a particular substrate varies dramatically.

To confirm that the cDNA encoded CE, the 1.7 kb *Eco*RI fragment was ligated into pCIneo to generate pCIRABFL and the plasmid transiently transfected into Cos7 cells. pCIneo contains the SV40 origin of replication allowing plasmid amplification in cells expressing the large T antigen, such as Cos7. The IC_{50} value for CPT-11 for cells expressing the CE was approximately 8-80 fold, and most typically about 56 fold, less than that of the parent cell line thus indicating that the enzyme has sensitized mammalian cells to CPT-11 (see Figure 5).

Rabbit CE has also been expressed in Spodoptera frugiperda S21 cells via a baculovirus vector. CE secreted in these cells was concentrated by ultrafiltration to approximately 1 ml containing approximately 30,000 micromoles/millimeter of enzyme activity.

Another aspect of the present invention relates to the ability of compositions of the present invention comprising a polynucleotide encoding a carboxylesterase and a disease-specific responsive promoter of selected tumor cells to sensitize the tumor cells to a chemotherapeutic prodrug. The ability of the combination of a rabbit CE of the present invention and CPT-11 to sensitize human tumor cells to CPT-11 was examined. Experiments were first performed to confirm that the metabolite produced by the activity of a CE of the present invention is biologically active *in vitro*. Rh30 cells were then exposed to the products of each reaction for one hour and the percentage of growth inhibition was determined. As expected, Rh30 cells exposed to 1 to 5 units of CE that had been inactivated by heating produced no inhibition of cell growth. In contrast, reaction products of CPT-11 incubated with 1 to 5 units of active CE produced a 30-60% inhibition of cell growth. These data are consistent with the conversion of CPT-11 to SN-38 by CE in these cells.

The CE activity of extracts of the transfected cells was then determined. The IC_{50} values for CPT-11 in Rh30 rhabdomyosarcoma cells that had been stably transfected with a rabbit liver CE cDNA of the present invention or the pIRES vector alone were also determined. Cells transfected with the CE cDNA contained approximately 60-fold more CE activity than control cells. The IC_{50} of CPT-11 for Rh30pIRES cells (no CE cDNA) was 4.33×10^{-6} M while the IC_{50} for the $Rh30pIRES_{rabbit}$ cells was 5.76×10^{-7} M. Therefore, the transfected cells were more than 8-fold more sensitive to CPT-11. These data are consistent with an increased conversion of CPT-11 to SN-38 in the cells transfected with a CE of the present invention.

Experiments have also been conducted which demonstrate that a CE of the present invention is capable of converting

the inactive metabolite APC to SN-38. Structures of these compounds are shown in Figure 8. Figure 6 shows the results of experiments *in vitro* where APC is converted to SN-38 in a concentration-dependent manner by a rabbit CE of the present invention. These data confirm the unique ability of a CE of the present invention to activate the prodrug CPT-11, as well as to activate one of its metabolites. Further, experiments in U-373 cells that express a CE of the present invention showed that these cells were sensitized to the growth inhibitory effects of APC (see Figure 7).

In vivo efficacy of the CE of the present invention to sensitize tumor cells to CPT-11 has also been demonstrated in two different types of tumor cells. Experiments conducted in a mouse model demonstrate that a CE of the present invention is capable of sensitizing cells to the growth inhibitory effects of CPT-11.

In a first set of experiments, the ability of rabbit CE to sensitize Rh30 rhabdomyosarcoma human tumor cells grown as xenografts in immune-deprived mice was demonstrated. In this preclinical model, expression of the transfected cDNA for rabbit CE was maintained for at least 12 weeks. Importantly, tumors were advanced (greater than 1 cm^3 in volume) before treatment with CPT-11 began. As depicted in Figure 9B, tumors in mice expressing CE and treated with 2.5 mg CPT-11/kg/day for five days each week for two weeks (one cycle of therapy), repeated every 21 days for a total of three cycles (over 8 weeks), regressed completely and did not regrow during the 12 weeks of the study. In contrast, tumors that did not express the CE regressed only transiently with CPT-11 treatment, with regrowth occurring within one week after CPT-11 treatment stopped (see Figure 9C).

In a second set of experiments, human U373 glioblastoma xenografts that express rabbit liver CE were shown to be more sensitive to CPT-11 than xenografts transfected with a control plasmid (no rabbit CE). Xenografts established from cells

transfected with the plasmid encoding rabbit CE regressed completely while xenografts from cells transfected with the control plasmid showed stable disease but no significant regression (see Figure 10).

Thus, these data support the use of the combination of polynucleotide encoding a CE of the present invention and CPT-11 to reduce the amount of CPT-11 needed to produce inhibition of tumor cell growth, or to sensitize the tumor cells to CPT-11. These data also support the use of the present invention to allow for decreased dosage with CPT-11 in cancer patients, thus reducing the likelihood of dose-limiting toxicity. Further, as shown by these experiments, APC, which is relatively nontoxic, can also be used as a chemotherapeutic prodrug in combination with a CE of the present invention to produce tumor-specific cell death while minimizing toxic side effects.

The present invention thus also relates to a method for treating cancer with reduced side effects. In one embodiment, a polynucleotide of the present invention is inserted into a viral vector using a gene transfer procedure. Preferred viral vectors include, but are not limited to, retroviral, adenoviral, herpesvirus, vaccinia viral and adeno-associated viral vectors. In this embodiment, it is preferred that the vector further comprise a disease-specific responsive promoter. The vectors can then be injected into the site of tumor removal along with systemic administration of a prodrug such as CPT-11 to inhibit the recurrence of tumors due to residual tumor cells present after surgical resection of a tumor.

Alternatively, the viral vector can be used to purge bone marrow of contaminating tumor cells during autologous transplant. Bone marrow purging via a viral vector such as adenovirus which expresses a CE of the present invention is performed *ex vivo*. Efficiency of removal of contaminating tumor cells is determined by PCR assays of purged samples. Data indicate that the method of the present invention is applicable to an animal model for purging bone marrow of

neuroblastoma cells such as that described in Example 6. Methods for preparation of the vectors, modes of administration, and appropriate doses of prodrug are well known to those of skill in the art. Other methods of gene delivery such as chemical and liposome-mediated gene transfer, receptor-mediated DNA uptake, and physical transfer by gene guns or electroporation may also be employed.

Another method for delivering CEs to selected tumor cells involves antibody direct enzyme prodrug therapy (ADEPT). In this method, human tumors are targeted by conjugation of tumor-specific marker antibody with a molecule such as rabbit liver CE. Cellular internalization of the complex and release of active CE would be achieved, leading to CPT-11 activation that is specific for cells expressing the marker antigen. Since the array of marker molecules expressed upon the cell surface is different for each tumor type, markers specific for each targeted tumor type can be selected as appropriate. Similarly, the use of avidin-biotin conjugated molecules to target tumor cells (Moro, M. et al. 1997. *Cancer Res.* 57:1922-1928) is also applicable for localization of CEs to the cell surface followed by drug activation at the targeted cell.

The rabbit liver CE is localized in the endoplasmic reticulum. Removal of the six terminal amino acids results in secretion of active protein into the extracellular milieu. Both the secreted and the endoplasmic reticulum-localized protein can convert CPT-11 to SN-38; therefore, the potential exists for a bystander effect from cells expressing the secreted enzyme. A similar bystander effect has been demonstrated for other enzyme/prodrug combinations, such as HSVtk and ganciclovir (Dilber, M.S. et al. 1997. *Cancer Res.* 57:1523-1528), and results in increased cytotoxicity. Extracellular activation of CPT-11 may result in more efficient eradication of MRD in that uninfected neighboring tumor cells would be killed by exogenously produced SN-38. Gene therapy protocols with a secreted CE in combination with CPT-11 may therefore be more appropriate for the elimination of residual tumor tissue. Accordingly, in this embodiment,

it may be preferred to use a fragment of a polynucleotide encoding a polypeptide which is secreted. For example, for rabbit liver, a cDNA encoding a protein which does not contain the six terminal amino acids depicted in Figure 4, or a cDNA encoding a rabbit liver CE enzyme consisting of amino acids 1-543 (SEQ ID NO:26) of Figure 4, may be preferred. Additionally, recent reports indicate that the tethering of drug activating enzymes to the extracellular cell surface can result in anti-tumor activity in human tumor xenografts when combined with appropriate prodrug (Marais, R. et al. 1997. *Nature Biotech.* 15:1373-1377). A tethered enzyme generates a local bystander effect since the protein is not free to circulate in the plasma. Attachment of a CE of the present invention to the cell surface should result in local extracellular activation of CPT-11 to SN-38 and enhance local cell kill. Purging bone marrow of contaminating tumor cells will be accomplished by an intracellular enzyme, whereas eradication of MRD is better achieved by an enzyme that activates CPT-11 at an extracellular location.

CEs of the present invention cleave the COOC bond present as an ester linkage in CPT-11 to generate SN-38 (see Figure 8). Since this enzyme may also catalyze the activation of other compounds that contain such a linkage, the present invention also provides assays for screening for compounds that contain this and related moieties. In one embodiment, the assay of the present invention is conducted in a cell systems using, for example, yeast, baculovirus, or human tumor cell lines. In this embodiment, compounds activated by CE will be identified and assessed for anticancer activity by growth inhibition or clonogenic cell survival assays using cells expressing or lacking a CE of the present invention. Alternatively, compounds can be screened in cell-free assays using a CE of the present invention isolated from host cells expressing this enzyme. In this embodiment, the ability of the enzyme to cleave a COOC ester linkage of a candidate compound is measured directly in a standard enzyme assay buffer system containing a CE of the present invention. Known

concentrations of candidate compounds can be added to assay tubes containing a biological buffer such as HEPES at pH 7.4 and the enzyme and incubated at 37°C for a selected amount of time. The reaction is then terminated by addition of methanol. The assay tubes are then centrifuged and the supernatant analyzed for the presence of cleaved compound fragment. Analysis of the supernatant can be performed by any number of well known techniques including, but not limited to, spectrofluorometric analysis, high pressure liquid chromatography or mass spectrometry. Compounds identified in these screening assays as potential anticancer prodrugs may require chemical modification for optimize their anti-tumor activity.

The following non-limiting examples are provided to further illustrate the claimed invention.

EXAMPLES

Example 1: Identification of CEs

A CE enzyme suitable for converting CPT-11 to the active form, SN-38 was identified by testing a variety of samples. This screening included enzymes from a series of sera, cell extracts and commercially available CEs using a rapid fluorometric assay. Certain of these enzymes show activity in metabolism of CPT-11.

Since partially purified CEs were commercially available, several of these were also tested for their ability to metabolize CPT-11. Both rabbit and pig liver CEs metabolized CPT-11 efficiently. The commercially available pig CE contained several proteins. However, the major bands were very similar in molecular weight and did not separate using SDS-PAGE. In contrast, the rabbit preparation consisted of only one major and one minor protein. Therefore, the rabbit proteins were chosen for further study.

The rabbit proteins were subjected to automated N-terminal amino acid sequencing. Both bands yielded protein sequences indicating that the peptides were not N-terminally blocked. The derived amino acid sequences were analyzed by

computer searches using the Fasta and BLAST comparison programs. Band 1 (approximately 60 kDa) demonstrated significant homology with several CE sequences, including a rabbit CE, present in the GenBank and Swissprot databases (Figure 1). However, the nucleic acid sequence encoding rabbit CE protein has not been disclosed. In addition, comparison of the amino acid sequence of the polypeptide encoded by the cDNA of the present invention with the published amino acid sequence for rabbit CE showed three mismatches. Further, the polypeptide encoded by the cDNA of the present invention contains an 8 amino acid insert and an 18 amino acid leader sequence at the N-terminus which the published sequence does not contain. Thus, the published amino acid sequence of a rabbit liver carboxylesterase protein (Swissprot Accession Number P12337; Korza, G. and J. Ozols. 1988. *J. Biol. Chem.* 263:3486-3495) is different from the polypeptide encoded by the cDNA of the present invention.

Example 2: Cloning of rabbit carboxylesterase

The cDNA encoding the rabbit CE protein of the present invention was isolated by synthesizing degenerate oligonucleotides from amino acid residues 1-5 (SEQ ID NO:6) and 518-524 (SEQ ID NO:10) of the published protein sequence of a rabbit liver CE (Korza, G. and J. Ozols. 1988. *J. Biol. Chem.* 263:3486-3495). The oligonucleotides constructed are shown in Figure 2. To amplify the rabbit cDNA by PCR, cDNA was prepared from rabbit liver poly A+ mRNA and multiple samples were prepared that contained the combination of oligonucleotide primers. Following heating at 95°C for five minutes, the polymerase was added at the annealing temperature and reactions cycled as follows: 94°C 45 seconds, annealing temperature (46-58°C) 1 minute, 72°C 90 seconds. Typically, 25 cycles of amplification were performed. A single product was obtained from one set of reactions that upon DNA sequencing was shown to encode a novel rabbit CE.

Since this represented a partial cDNA, both 5' and 3' RACE were used to amplify the entire coding sequence. Unique primers of 27 and 28 nucleotides, corresponding to the 5' and 3' ends respectively, were designed from the partial DNA sequence. These oligonucleotides were used in combination with the AP1 primer to amplify sequences prepared from Marathon adapted rabbit liver cDNA. Touchdown PCR (Don, R.H. et al. 1991. *Nucleic Acids Res.* 19:4008) was performed as according to the Marathon cDNA amplification protocol. A single product of approximately 420 bp was generated by the 3' primer, however no product was observed with the 5' oligonucleotide. Standard PCR amplification protocols (94°C 45 seconds, 60°C 1 minute, 72°C 1 minute, 30 cycles) resulted in a smear of DNA products with a minor band at approximately 280 bp. Attempts to increase the specificity of the reaction were unsuccessful. Therefore, DNA was isolated from the agarose gels and then ligated into pCRII-TOPO. DNA sequencing indicated the presence of the oligonucleotide RACE primers in both samples. The 3' RACE product extended 407 bp from the specific primer and encoded the terminal amino acids consistent with the published data (Korza, G. and J. Ozols. 1988. *J. Biol. Chem.* 263:3486-3495). In addition, a poly A tail was present and the original Marathon cDNA synthesis primer sequences could be identified. The 5' RACE product extended 247 bp from the CE specific primer and encoded the published amino acid sequence. An additional 18 residue hydrophobic leader sequence beginning with a methionine initiation codon was identified, consistent with the amino acids present at the N-termini of CEs derived from other species (Figure 3). The entire transcript including both untranslated 5' and 3' sequences, as determined by the RACE experiments, was 1886 nt long, very similar to that indicated by the Northern analysis. This confirmed that the cDNA described in these experiments was full length.

To amplify a full length rabbit CE cDNA, oligonucleotide primers RabNTERM (GGCAGGAATTCTGCCATGTGGCTCTG; SEQ ID NO:23) and RabCTERM (CGGGAATTCACATTCACAGCTCAATGT; SEQ ID NO:24) were

designed to create *Eco*RI sites 9 bp upstream of the ATG initiation codon and 8 bp downstream of the TGA termination codon. These were used to amplify rabbit liver cDNA using *Pfu* polymerase. The initial 5 cycles of amplification were performed as follows: 94°C, 45 seconds; 50°C, 1 minute; 72°C, 90 seconds with the annealing temperature raised to 56°C for the subsequent 25 cycles. This allowed the formation of the *Eco*RI restriction sites at the termini of the cDNA. A product of approximately 1700 bp was obtained, ligated into pUC9 restricted with *Eco*RI and the entire DNA was sequenced.

Example 3: Expression of rabbit CE in Spodoptera frugiperda Sf21

Cells (4 x 10^7) were plated in the lower chamber of an Integra CL1000 flask (Integra Biosciences, Ijamsville, MD) in 45 mls of Insect Xpress media (BioWhittaker, Walkersville, MD). To ensure adequate growth of the cells, 500 mls of complete Grace's media was added to the upper chamber of the flask. After incubation at 27°C for 2 days, baculovirus were added to the cells in the lower chamber at a multiplicity of infection of 20. Media in the lower chamber was assayed every 24 hours for carboxylesterase (CE) activity and usually harvested after 120 hours. The secreted CE was concentrated by ultrafiltration to yield approximately 1 ml of sample containing approximately 30,000 micromoles/ml of enzyme activity.

Example 4: *In vitro* biological activity of rabbit CE

The *in vitro* activity of rabbit liver CE was examined in tumor cell lines. The growth inhibition of CPT-11 was compared in cells with and without active rabbit CE. The cells used were Rh30 cells (10^7) that had been electroporated with 20 μg of IRES plasmid DNA or plasmid containing CE cDNA in a volume of 200 μl of phosphate buffered saline. Optimized conditions for electroporation were achieved using 180 V and 960 uF. The cells were plated into 75 cm^2 flasks in fresh

media and 500 μg G418/ml added 48 hours following transfection to select for cells expressing the neo gene and the CE. Cells were grown for a minimum of 10 days before use in growth inhibition experiments.

In the first assay, CPT-11 was pre-incubated with rabbit liver CE to produce SN-38 prior to exposure of the cells to drug. Specifically, 0.5 to 5 units of CE were incubated with 1 μM CPT-11 at 37°C in DMEM medium for 2 hours. Each reaction mixture was then filter-sterilized and Rh30 cells were exposed to drug for one hour, at which time the medium was replaced with drug-free medium containing serum. Enzyme that had been inactivated by boiling for five minutes prior to incubation with drug or CPT-11 to which no enzyme had been added were used as negative controls. Cells were allowed to grow for 3 cell doubling times and cell numbers were determined.

In the second type of growth inhibition assay, Rh30 cells that had been transfected with either pIRES parent plasmid DNA or the plasmid containing the rabbit CE cDNA were exposed to different concentrations of CPT-11. Drug was added to tissue culture medium of each of the stably transfected cell lines for two hours, after which time the medium was replaced with drug-free medium. Cells were then allowed to grow for 3 cell doublings as before. Results were expressed as the concentration of drug required to reduce cell growth to 50% of control cells, or IC_{50}.

Results showed that extracts of the transfected cells contained greater than 60-fold more CE activity than controls as determined by the conversion of o-nitrophenyl acetate to o-nitrophenol. Further, the Rh30pIRES cells transfected with rabbit CE were greater than 8-fold more sensitive to CPT-11 than controls, as shown by a decrease in the IC_{50} values. Therefore, Rh30 cells stably transfected with rabbit CE were more sensitive to growth inhibition by CPT-11 than cells that did not contain the cDNA for rabbit CE.

Example 5: Rabbit CE activates APC, a novel prodrug

In addition to efficiently converting CPT-11 to the active compound SN-38, experiments were also performed demonstrating the ability of rabbit liver CE to convert the inactive metabolic end product APC to SN-38. No known human enzyme activates APC. Figure 6 shows the kinetics of conversion of APC to SN-38 by 50 units of rabbit liver CE in an *in vitro* reaction. Figure 7 shows that U-373 glioma cells that express the rabbit liver CE, but not human alveolar macrophage carboxylesterase which is 85% homologous to the rabbit enzyme, are sensitized to the growth inhibitory effects of APC. Thus, the combination of APC and sensitization of selected tumor cells with rabbit liver CE as described above can be used to produce a tumor-specific cell death while greatly minimizing the toxic side effects associated with administration of chemotherapy.

Example 6: Use of rabbit CE in an *in vivo* model for MRD

A xenograft model for MRD has been developed to demonstrate the effectiveness of the combination of rabbit CE and prodrug in the prevention of MRD. In this model, treatment of immune-deprived mice, i.e., SCID mice, bearing human NB-1691 xenografts with 10 mg/kg CPT-11 daily for 5 days on two consecutive weeks results in complete regression of the tumor. However, within 4-6 weeks, tumors are palpable in the exact position where the original xenograft was implanted. Since these tumors arise from cells that survived the initial cycle of chemotherapy, this model therefore mimics results seen in patients following surgical resection of the primary tumor and subsequent regrowth at the same site.

Experiments were performed in this model to compare the responses of mice bearing human Rh30 and Rh30pIRES$_{rabbit}$ xenografts. Rh30 rhabdosarcoma xenografts were transfected with pIRESneo plasmid containing the cDNA for rabbit liver CE and selected with G418. Expression of CE was confirmed by

biochemical assay using the CE substrate o-NPA and maintained for at least 12 weeks. Two groups of SCID mice were then injected with the transfected $Rh30pIRES_{rabbit}$ cells subcutaneously into the flanks. A third group of control mice was injected in identical fashion with Rh30 cells not transfected with the plasmid. When the tumors reached a size of approximately 1 cm^3, 2.5 mg CPT-11/kg/day was administered five days each week for two weeks (one cycle of therapy), repeated every 21 days for a total of three cycles (over 8 weeks) to one group of mice injected with the transfected $Rh30pIRES_{rabbit}$ cells and the third group of control mice.

The tumors expressing rabbit CE regressed completely and did not regrow during the 12 weeks of the study (Figure 9B). In contrast, tumors not expressing the CE regressed only transiently, regrowing within one week after CPT-11 treatment had stopped (Figure 9C).

Similar studies were performed employing U373 glioblastoma cells transfected with the pIRESneo plasmid or with pIRESneo containing the cDNA for rabbit liver CE and selected with G418. Expression of CE in the tumor cells was confirmed by biochemical assay using the substrate o-NPA. Cells were injected subcutaneously into the flanks of the SCID mice. When tumors reached approximately 1 cm^3 in size, CPT-11 was administered daily for five days each week as described above, for three cycles, at a dose of 7.5 mg/kg/day.

The U373 cells that expressed rabbit CE were also more sensitive to CPT-11. Xenografts established from cells transfected with the plasmid encoding rabbit CE regressed completely while xenografts from cells transfected with the control plasmid showed stable disease with no significant regression. These data in two different human tumor cell types demonstrate the *in vivo* efficacy of rabbit CE to sensitize tumor cells to CPT-11.

Adenovirus expressing the rabbit CE under control of a tumor-specific promoter administered subcutaneously at the

site of xenograft implantation in this model during the 4 to 6 week period when tumors are not present, followed by treatment with low doses of CPT-11, also demonstrates the effectiveness of the virus at preventing MRD. Typically, since tumor regression is complete 3 weeks after commencing treatment with CPT-11, adenovirus/drug administration begins at week 4. In initial experiments, adenovirus is administered on Monday, Wednesday, Friday and CPT-11 is given daily on Tuesday through Saturday for two cycles. This permits determination of the most tolerated, effective schedule and dosage of adenovirus and CPT-11 administration to produce the longest delay of recurrent disease. These results are used to determine correct dosage for treatment of human MRD. The starting point for the animal experiments is injection of 10^5 to 10^8 pfu of adenovirus containing the rabbit CE of the present invention.

Example 7: Use of rabbit CE/prodrug to purge bone marrow of tumor cells

Intravenous injection of human neuroblastoma NB-1691 tumor cells into immune-deprived mice results in the development of widespread metastatic disease with death occurring on days 36-38. Since both synaptophysin and tyrosine hydroxylase expression are specific for neuroblastoma cells, RT/PCR analysis of these mRNAs can detect tumor cells present in mixed populations of cells. Circulating neuroblastoma cells can be detected in the peripheral blood of these animals 36 days after injection with NB-1691. Studies will then determine whether the bone marrow of these same animals contains neuroblastoma cells. The success of *ex vivo* purging of bone marrow with the rabbit liver CE/CPT-11 combination is demonstrated by transplanting purged bone marrow into lethally irradiated mice. If mice remain disease free for extended periods of time, this indicates that the adenoviral CE/prodrug purging therapy kills neuroblastoma cells in the donor marrow.

Example 8: Treatment of Minimal Residual Disease (MRD) in humans

The rabbit CE in combination with CPT-11 or other prodrugs activated by this enzyme is used to purge bone marrow of residual tumor cells prior to autologous bone marrow transplants to prevent recurrence of local MRD following removal of bulk tumor by surgery or chemotherapy. Following debulking of the primary tumor, adenovirus containing the rabbit liver CE under the control of a tumor-responsive promoter is applied to the tumor margins at either the time of surgery, by stereotaxic injection, or by implantation of a time-release polymer or other material. Anti-tumor effect of single application at time of surgery is compared with the effect produced by repetitive or time-release use of adenoviral constructs. Adenovirus dose ranges from 10^6 to 10^{10} plaque-forming units as has been reported to be effective for intratumoral injection of adenovirus (Heise, C. et al. 1977. *Nature Med.* 3:639-645). CPT-11 is administered over the next one to six weeks to elicit tumor selective cell kill. Doses and schedules of CPT-11 are determined in clinical trials of CPT-11 by itself and in human xenograft model systems to produce maximal tumor effect.

Example 9: Purging bone marrow of tumor cells in humans

Tumor cells that contaminate bone marrow used for autologous transplant contribute to relapse of disease. Therefore, the rabbit liver CE is used in combination with a suitable prodrug to eradicate tumor cells in marrow samples to be used for transplant. This approach maintains the viability of hematopoietic cells required for reconstitution. Bone marrow samples are transduced *ex vivo* with adenovirus containing the rabbit liver CE cDNA, using a multiplicity of infection (moi) that will infect 100% of the tumor cells. Typically, a moi of 0.5 to 10 is adequate for tumor cells, while a moi of 100 to 1,000 is required to transduce a majority of hematopoietic progenitor cells. Two days

following adenoviral transduction, cells are exposed for two hours to a range of CPT-11 concentrations, usually varying from 50 nM to 100 μM. Two days after exposure to drug, the marrow sample is harvested and stored for reinfusion into the patient and reconstitution of a tumor-free marrow.

What is claimed is:

1. An isolated polynucleotide encoding a carboxylesterase capable of metabolizing a chemotherapeutic prodrug and inactive metabolites thereof to active drug.

2. The isolated polynucleotide of claim 1 consisting of a cDNA of Figure 4 (SEQ ID NO:20).

3. The isolated polynucleotide of claim 1 consisting of a cDNA encoding a carboxylesterase consisting of amino acids 1-543 of Figure 4 (SEQ ID NO:26).

4. An isolated polynucleotide capable of hybridizing with a polynucleotide of claim 1.

5. A vector comprising the polynucleotide of claim 1.

6. A host cell comprising the vector of claim 5.

7. A polypeptide encoded by the polynucleotide of claim 1.

8. A composition comprising the polynucleotide of claim 1 and a disease-specific responsive promoter.

9. The composition of claim 8 wherein said disease-specific responsive promoter is a *myc* promoter.

10. The composition of claim 9 wherein the myc promoter is ODC.

11. A method for sensitizing tumor cells to a chemotherapeutic prodrug comprising transfecting selected tumor cells with the composition of claim 8.

12. A method of inhibiting tumor cell growth comprising:

(a) sensitizing tumor cells in accordance with the method of claim 11; and

(b) contacting said sensitized tumor cells with a chemotherapeutic prodrug so that tumor cell growth is inhibited.

13. The method of claim 12 wherein the chemotherapeutic prodrug is selected from a group consisting of CPT-11 and APC.

14. A method of inhibiting tumor recurrence in a patient comprising:

(a) surgically removing a tumor from a patient;

(b) administering the composition of claim 8 at the site of tumor resection; and

(c) administering a chemotherapeutic prodrug systemically so that tumor recurrence is inhibited.

15. The method of claim 14 wherein the chemotherapeutic prodrug is selected from a group consisting of CPT-11 and APC.

16. A method of purging bone marrow cells of tumor cells comprising:

(a) removing bone marrow cells from a patient; and

(b) contacting the bone marrow cells with the composition of claim 8 and a chemotherapeutic prodrug.

17. A method of inhibiting tumor growth in a patient comprising administering to a patient a composition of claim 8 and APC.

18. A drug screening assay for identifying drugs that are activated by a carboxylesterase enzyme comprising:

(a) transfecting cells in culture with the polynucleotide of claim 1;

(b) contacting said cells with a candidate drug; and

(c) determining growth or survival of said cells in the presence of the candidate drug.

19. A drug screening assay for identifying compounds containing a COOC ester linkage that are activated by a carboxylesterase enzyme comprising:

(a) adding a known concentration of a test compound containing a COOC ester linkage to an assay tube containing a biological buffer and a polypeptide of claim 7;

(b) incubating the assay tubes; and

(c) analyzing contents of the assay tube for cleavage fragments of the test compound at the COOC ester linkage wherein the presence of the cleavage fragment is indicative of activation of the compound by the carboxylesterase enzyme.

AMENDED CLAIMS

[received by the International Bureau on 05 August 1999 (05.08.99);
new claims 20, 21 and 22 added; remaining claims unchanged (1 page)

19. A drug screening assay for identifying compounds containing a COOC ester linkage that are activated by a carboxylesterase enzyme comprising:

(a) adding a known concentration of a test compound containing a COOC ester linkage to an assay tube containing a biological buffer and a polypeptide of claim 7;

(b) incubating the assay tubes; and

(c) analyzing contents of the assay tube for cleavage fragments of the test compound at the COOC ester linkage wherein the presence of the cleavage fragment is indicative of activation of the compound by the carboxylesterase enzyme.

20. A method for delivering carboxylesterases to selected tumor cells comprising:

(a) selecting an antibody specific for a marker on the selected tumor cells;

(b) conjugating the tumor-specific marker antibody to the carboxylesterase to form a complex; and

(c) administering the complex so that the carboxylesterases are delivered to the selected tumor cells.

21. A method of inhibiting growth of selected tumor cells in a patient comprising delivering to the selected tumor cells in the patient carboxylesterases in accordance with the method of claim 20.

22. The method of claim 21 further comprising administering to the patient a chemotherapeutic prodrug and inactive metabolites thereof which are metabolized to active drug by carboxylesterases.

```
Residue#

Rabbit AA                                HPSAPVXVDTVHGKVLGKFVSXEGFAQPVAKFXG
                                         ||||| |||| |||||||||| |||||||| | |
Rabbit     (P12337)                      HPSAPPVVDTVKGKVLGKFVSLEGFAQPVAVFLGVP

Human      (P23141)  MWLRAFILATLSASAAWGHPSSPPVVDTVHGKVLGKFVSLEGFAQPVAIFLGIP

Rat        (P10959)  MWLCALVWASLAVCPIWGHPSSPPVVDTTKGKVLGKYVSLEGFTQPVAVFLGVP

Mouse      (P23953)  MWLHALVWASLAVCPILGHSLLPPVVDTTQGKVLGKYISLEGFEQPVAVFLGVP
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FIGURE 1

Residue #	1				5
Amino acid sequence	His	Pro	Ser	Ala	Pro
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		C		C	
	T		T		
Oligonucleotide Rab51	CAC	CCI	AGC	GCI	CC
	T		T		
Amino acid sequence	His	Pro	Ser	Ala	Pro
Coding Sequence	CAC	CCA	AGC	GCA	CC
	T	G	T	G	
		C		C	
	T		T		
Oligonucleotide Rab 52	CAC	CCI	TCI	GCI	CC

FIGURE 2

Residue #	518						
Amino acid sequence	Ala	Phe	Trp	Thr	Glu	Leu	Trp
Coding sequence	GCA	TTC	TGG	ACA	GAA	CTA	TGG
	G	T		G	G	G	
	C			C		C	
	T			T		T	
Reverse complement	CCA	AAG	TTC	AGT	CCA	GAA	AGC
		G	C	G		A	G
		C		C			C
		T		T			T
Oligonucleotide Rab 31	CCA	IAG	TTC	IGT	VVA	GAA	IGC
			C			A	
Amino acid sequence	Ala	Phe	Trp	Thr	Glu	Leu	Trp
Coding sequence	GCA	TTC	TGG	ACA	GAA	CTA	TGG
	G	T		G	G	G	
	C			C			
	T			T			
Reverse compliment	CCA	TAA	TTC	AGT	CCA	GAA	AGT
		C	C	G		A	G
				C			C
				T			T
Oligonucleotide	CCA	TAA	TTC	IGT	CCA	GAA	IGC
		C	C			A	

Residue #		
Rabbit		*MWLCALALASLAACTAWGHPSAPPVVDTVK*
Rat	(P10959)	MWLCALVWASLAVCPIWGHPSSPPVVDTTK
Human	(P23141)	MWLRAFILATLSASAAWGHPSSPPVVDTVH
Rat	(P16303)	MRLYPLVWLFLAACTAWGYPSSPPVVNTVK
Mouse	(P23953)	MWLHALVWASLAVCPILGHSLLPPVVDTTQ

FIGURE 3

```
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Pro Pro Val Val Asp Thr Val His Gly Lys Val Leu Gly Lys Phe Val Ser Leu Glu Gly Phe Ala Gln Pro Val

GCC GTC TTC CTG GGA GTC CCC TTC GCC AAG CCC CCT CTT GGA TCC CTG AGG TTT GCA CCA CCA CAG CCT GCA GAA
Ala Val Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe Ala Pro Pro Gln Pro Ala TCA

TTG AGC CAC GTG AAG AAC ACC ACC TCC TAC CCT CCC ATG TGC TCC CAG GAC GCA GTA TCA GGG CAT ATG CTC Glu
Ser Trp Ser His Val Lys Asn Thr Thr Ser Tyr Pro Pro Met Cys Ser Gln Asp Ala Val Ser Gly His Met TCG
GAG CTC TTC ACC AAC AGA AAA GAG AAC ATC CCT CTT AAG TTT TCT GAA GAC TGC CTT TAC CTG AAT ATT TAC
Leu Ser Glu Leu Phe Thr Asn Arg Lys Glu Asn Ile Pro Leu Lys Phe Ser Glu Asp Cys Leu Tyr Leu Asn Ile

ACC CCT GCT GAC CTG ACA AAG AGA GGC AGG CTG CCG GTG ATG GTG TGG ATC CAT GGA GGT GGT CTG ATG GTG GGT
Tyr Thr Pro Ala Asp Leu Thr Lys Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Met Val

GGA GCA TCA ACC TAT GAT GGC CTG GCT CTT TCT GCC CAT GAG AAC GTG GTG GTG GTG ACC ATT CAG TAC CGC CTG
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GGC ATC TGG GGA TTC TTC AGC ACA GGA GAT GAG CAC AGC CGA GGG AAC TGG GGT CAC TTG GAC CAG GTG GCT GCG
Leu Gly Ile Trp Gly Phe Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp Gln Val Ala

CTG CGG TGG GTC CAG GAC AAC ATT GCC AAC TTT GGA GGG GAC CCA GGC TCT GTG ACC ATC TTT GGA GAG TCA GCA
Ala Leu Arg Trp Val Gln Asp Asn Ile Ala Asn Phe Gly Gly Asp Pro Gly Ser Val Thr Ile Phe Gly Glu Ser
```

FIGURE 4

```
GGA GGT CAA AGT GTC TCT ATC CTT CTA TTA TCC CCC CTG ACC AAG AAT CTC TTC CAT CGA GCA ATT TCC GAG AGT
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GGC GTG GCC CTC CTT TCC AGT CTC TTC AGG AAG AAC ACC AAG TCC TTG GCT GAG AAA ATT GCC ATC GAA GCT GGG
Ser Gly Val Ala Leu Leu Ser Ser Leu Phe Arg Lys Asn Thr Lys Ser Leu Ala Glu Lys Ile Ala Ile Glu Ala

TGT AAA ACC ACC ACC TCG GCT GTC ATG GTT CAC TGC CTG CGC CAG AAG ACA GAG GAA GAA CTC ATG GAG GTG ACA
Gly Cys Lys Thr Thr Thr Ser Ala Val Met Val His Cys Leu Arg Gln Lys Thr Glu Glu Glu Leu Met Glu Val

TTG AAA ATG AAA TTT ATG GCT CTA GAT CTA GTT GGC GAC CCC AAA GAG AAC ACC GCC TTC CTG ACC ACT GTG ATT
Thr Leu Lys Met Lys Phe Met Ala Leu Asp Leu Val Gly Asp Pro Lys Glu Asn Thr Ala Phe Leu Thr Thr Val

GAT GGG GTG CTG CTG CCA AAA GCA CCT GCA GAG ATT CTG GCA GAG AAG AAA TAC AAC ATG CTG CCC TAC ATG GTG
Ile Asp Gly Val Leu Leu Pro Lys Ala Pro Ala Glu Ile Leu Ala Glu Lys Lys Tyr Asn Met Leu Pro Tyr Met

GGA ATC AAC CAG CAA GAG TTT GGC TGG ATT ATC CCA ATG CAA ATG CTG GGC TAT CCA CTC TCT GAA GGC AAA CTG
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Leu Asp Gln Lys Thr Ala Thr Glu Leu Leu Trp Lys Ser Tyr Pro Ile Val Asn Val Ser Lys Glu Leu Thr Pro

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FIGURE 4 CONTINUED

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GAG CTC TGG GCC AAG GAG GCA GCA AGG CCA CGT GAG ACA GAG CAC ATT GAG CTG TGA ATT GAATTC
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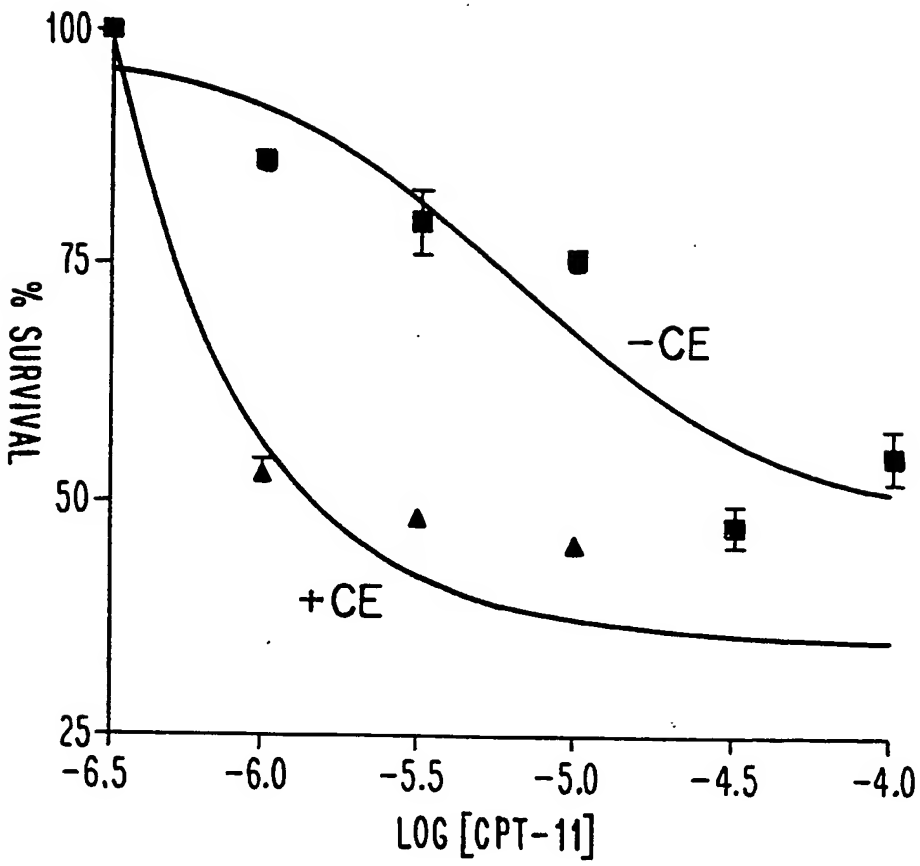
FIGURE 4 CONTINUED



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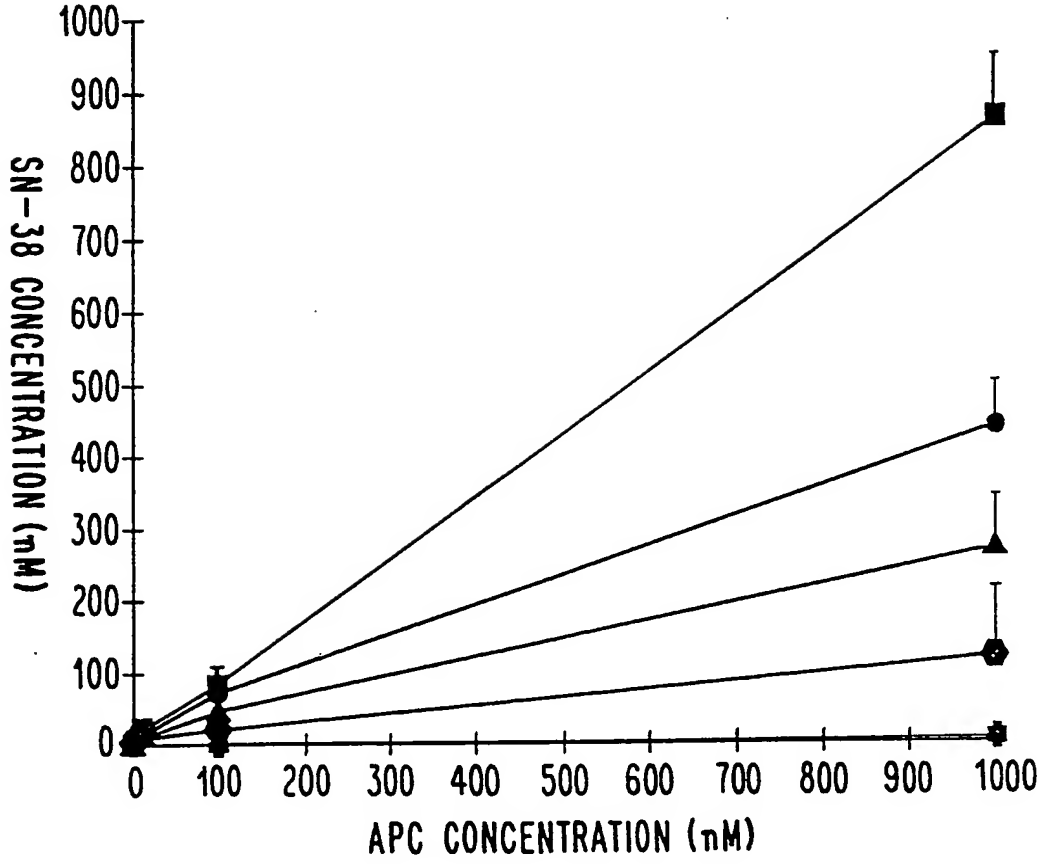
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Fig. 6

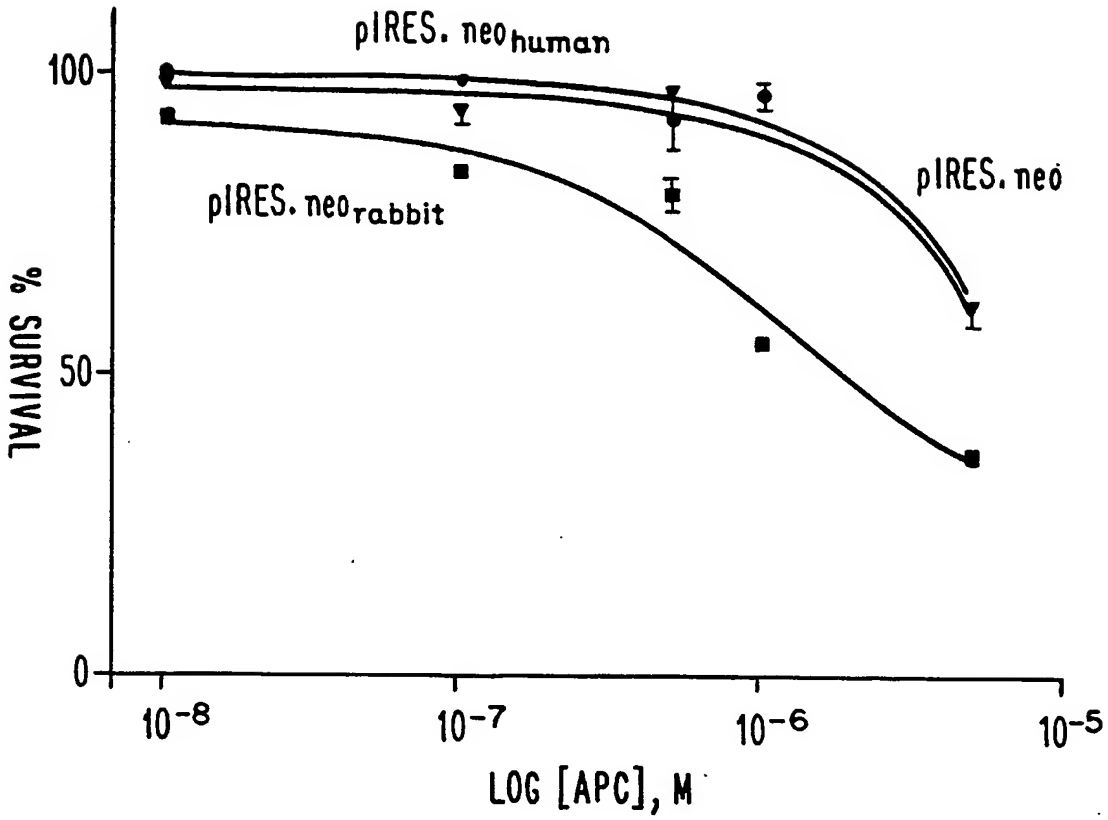
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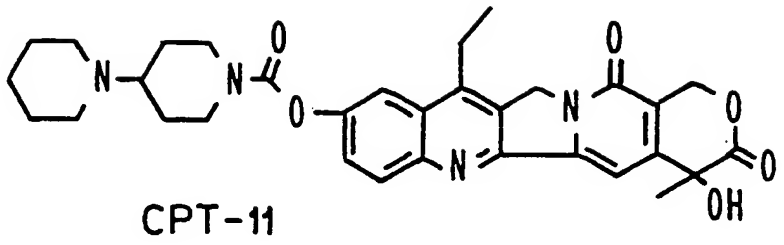
Fig. 7



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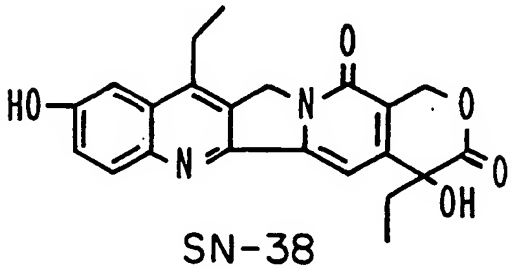

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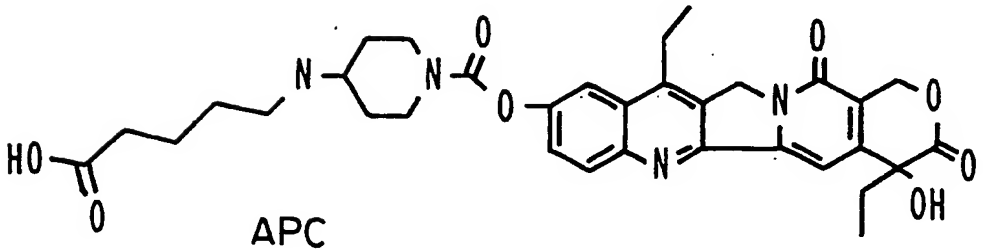
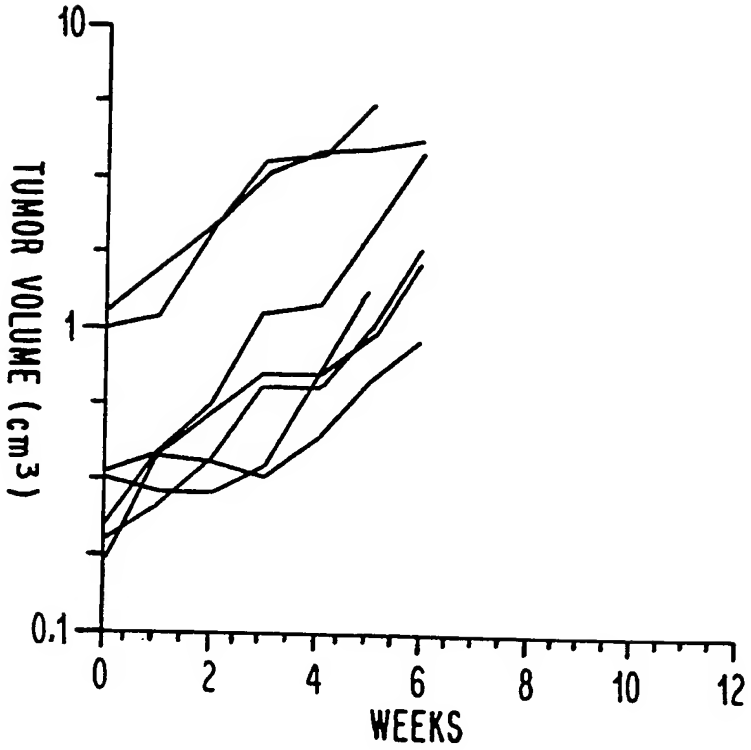


APC

Fig. 8

Fig. 9A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10
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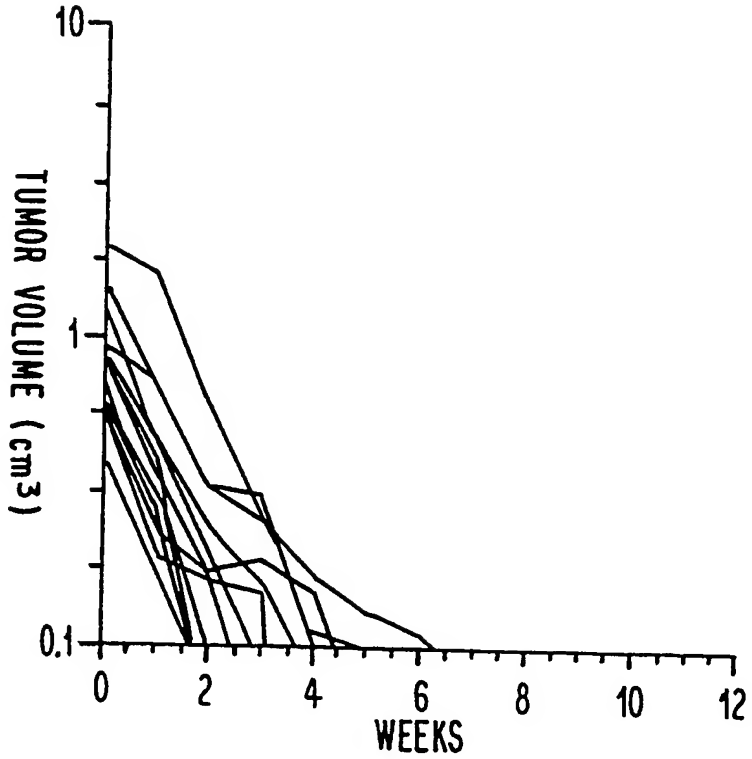
WEEKS

Fig. 9B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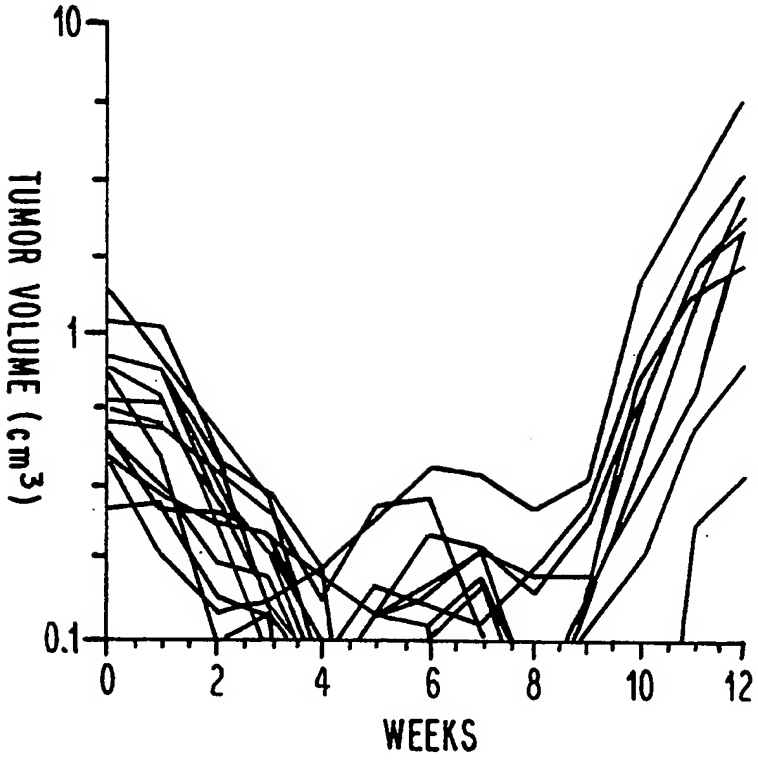

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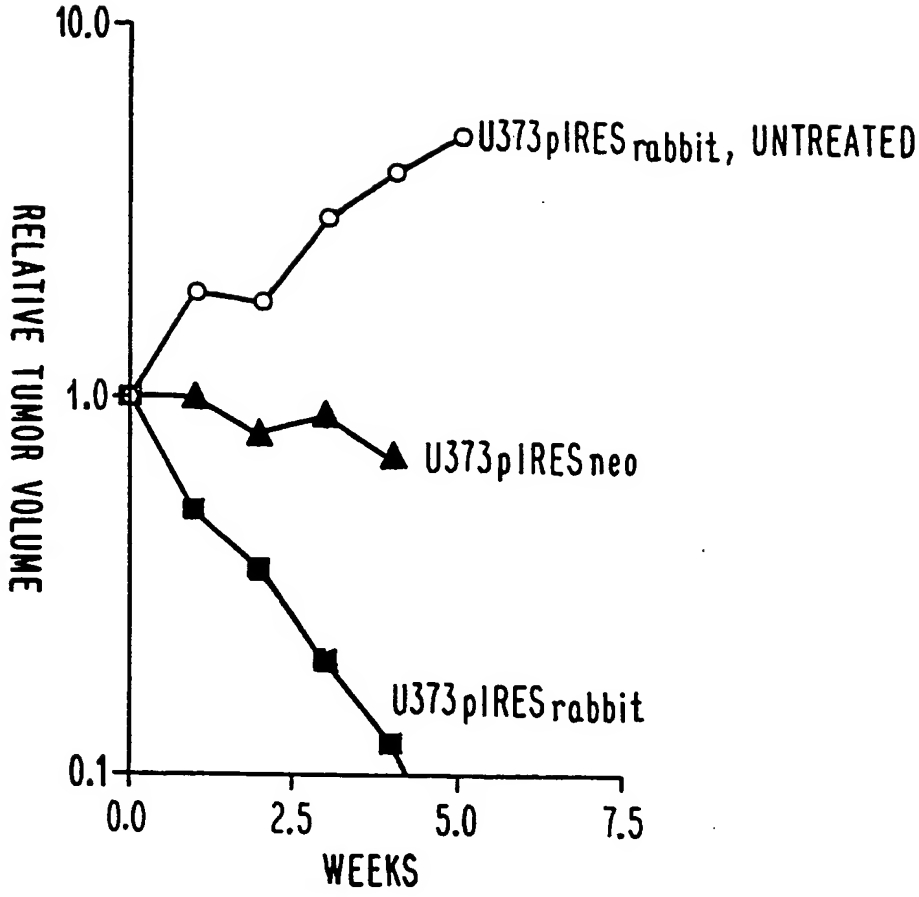
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Fig. 10

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Peter, Houghton J.

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35 40 45

Val Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg
50 55 60

Phe Ala Pro Pro Gln Pro Ala Glu Ser Trp Ser His Val Lys Asn Thr
65 70 75 80

Thr Ser Tyr Pro Pro Met Cys Ser Gln Asp Ala Val Ser Gly His Met
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Leu Ser Glu Leu Phe Thr Asn Arg Lys Glu Asn Ile Pro Leu Lys Phe

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Ser Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr
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Met Val Gly Gly Ala Ser Thr Tyr Asp Gly Leu Ala Leu Ser Ala His
145                 150                 155                 160

Glu Asn Val Val Val Val Thr Ile Gln Tyr Arg Leu Gly Ile Trp Gly
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Phe Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu
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Asp Gln Val Ala Ala Leu Arg Trp Val Gln Asp Asn Ile Ala Asn Phe
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Gly Gly Asp Pro Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly
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Gln Ser Val Ser Ile Leu Leu Leu Ser Pro Leu Thr Lys Asn Leu Phe
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His Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Leu Ser Ser Leu Phe
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Arg Lys Asn Thr Lys Ser Leu Ala Glu Lys Ile Ala Ile Glu Ala Gly
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Cys Lys Thr Thr Thr Ser Ala Val Met Val His Cys Leu Arg Gln Lys
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Thr Glu Glu Glu Leu Met Glu Val Thr Leu Lys Met Lys Phe Met Ala
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Leu Asp Leu Val Gly Asp Pro Lys Glu Asn Thr Ala Phe Leu Thr Thr
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Val Ile Asp Gly Val Leu Leu Pro Lys Ala Pro Ala Glu Ile Leu Ala
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Glu Lys Lys Tyr Asn Met Leu Pro Tyr Met Val Gly Ile Asn Gln Gln
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Glu Phe Gly Trp Ile Ile Pro Met Gln Met Leu Gly Tyr Pro Leu Ser
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Lys Tyr Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe
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Leu Asp Met Leu Ala Asp Leu Leu Phe Gly Val Pro Ser Val Asn Val
            420                 425                 430

Ala Arg His His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Tyr
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Arg Tyr Arg Pro Ser Phe Ser Ser Asp Met Arg Pro Lys Thr Val Ile
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Gly Asp His Gly Asp Glu Ile Phe Ser Val Leu Gly Ala Pro Phe Leu
465                 470                 475                 480

Lys Glu Gly Ala Thr Glu Glu Glu Ile Lys Leu Ser Lys Met Val Met
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Lys Tyr Trp Ala Asn Phe Ala Arg Asn Gly Asn Pro Asn Gly Glu Gly
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Leu Pro Gln Trp Pro Ala Tyr Asp Tyr Lys Glu Gly Tyr Leu Gln Ile
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Gly Ala Thr Thr Gln Ala Ala Gln Lys Leu Lys Asp Lys Glu Val Ala
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Phe Ala Pro Pro Gln Pro Ala Glu Ser Trp Ser His Val Lys Asn Thr
65                  70                  75                  80

Thr Ser Tyr Pro Pro Met Cys Ser Gln Asp Ala Val Ser Gly His Met
                85                  90                  95

Leu Ser Glu Leu Phe Thr Asn Arg Lys Glu Asn Ile Pro Leu Lys Phe
            100                 105                 110

Ser Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr
        115                 120                 125

Lys Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu
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Met Val Gly Gly Ala Ser Thr Tyr Asp Gly Leu Ala Leu Ser Ala His
145                 150                 155                 160

Glu Asn Val Val Val Val Thr Ile Gln Tyr Arg Leu Gly Ile Trp Gly
                165                 170                 175

Phe Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu
            180                 185                 190

Asp Gln Val Ala Ala Leu Arg Trp Val Gln Asp Asn Ile Ala Asn Phe
        195                 200                 205

Gly Gly Asp Pro Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly
    210                 215                 220

Gln Ser Val Ser Ile Leu Leu Leu Ser Pro Leu Thr Lys Asn Leu Phe
225                 230                 235                 240

His Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Leu Ser Ser Leu Phe
                245                 250                 255

Arg Lys Asn Thr Lys Ser Leu Ala Glu Lys Ile Ala Ile Glu Ala Gly
            260                 265                 270

Cys Lys Thr Thr Thr Ser Ala Val Met Val His Cys Leu Arg Gln Lys
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        275                 280                 285

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Leu Asp Leu Val Gly Asp Pro Lys Glu Asn Thr Ala Phe Leu Thr Thr
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Val Ile Asp Gly Val Leu Leu Pro Lys Ala Pro Ala Glu Ile Leu Ala
                325                 330                 335

Glu Lys Lys Tyr Asn Met Leu Pro Tyr Met Val Gly Ile Asn Gln Gln
            340                 345                 350

Glu Phe Gly Trp Ile Ile Pro Met Gln Met Leu Gly Tyr Pro Leu Ser
        355                 360                 365

Glu Gly Lys Leu Asp Gln Lys Thr Ala Thr Glu Leu Leu Trp Lys Ser
    370                 375                 380

Tyr Pro Ile Val Asn Val Ser Lys Glu Leu Thr Pro Val Ala Thr Glu
385                 390                 395                 400

Lys Tyr Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe
                405                 410                 415

Leu Asp Met Leu Ala Asp Leu Leu Phe Gly Val Pro Ser Val Asn Val
            420                 425                 430

Ala Arg His His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Tyr
        435                 440                 445

Arg Tyr Arg Pro Ser Phe Ser Ser Asp Met Arg Pro Lys Thr Val Ile
    450                 455                 460

Gly Asp His Gly Asp Glu Ile Phe Ser Val Leu Gly Ala Pro Phe Leu
465                 470                 475                 480

Lys Glu Gly Ala Thr Glu Glu Glu Ile Lys Leu Ser Lys Met Val Met
                485                 490                 495

Lys Tyr Trp Ala Asn Phe Ala Arg Asn Gly Asn Pro Asn Gly Glu Gly
            500                 505                 510

Leu Pro Gln Trp Pro Ala Tyr Asp Tyr Lys Glu Gly Tyr Leu Gln Ile
        515                 520                 525

Gly Ala Thr Thr Gln Ala Ala Gln Lys Leu Lys Asp Lys Glu Val
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530 535 540

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03171

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :536/23.2; 435/320.1, 252.3, 325, 197, 6, 19; 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.2; 435/320.1, 252.3, 325, 197, 6, 19; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, SCISEARCH, LIFESCI, BIOTECHDS, BIOSIS, EMBASE, HCAPL, NTIS, PATOSWO, PATOSEP, JAPIO, CANCERLIT search terms: carboxylesterase#, cpt-11 or irinotecan

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	SENTER, P.D. et al. The Role of Rat Serum Carboxylesterase in the Activation of Paclitaxel and Camptothecin Produgs. Cancer Research. 01 April 1996, Vol. 56, pages 1471-1474, see particularly pages 1473-1474.	7 --- 1, 4-6, 8-19
X - Y	ALEXSON, S.E.H. et al. Molecular Cloning and Identification of a Rat Serum Carboxylesterase Expressed in the Liver. J. Biol. Chem. 24 June 1994. Vol. 269, No. 25, pages 17118-17124, see entire article.	1-7 ---- 8-19
X - Y	SIGMA CHEMICAL COMPANY CATALOG, Product Numbers E2884 and E9636. 1994, pages 410-411, see entire document.	7 --- 1, 4-6, 8-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 JUNE 1999	Date of mailing of the international search report 07 JUL 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer REBECCA PROUTY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03171

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	MILLER, S.K. et al. Purification and Physical Properties of Oligomeric and Monomeric Carboxylesterases from Rabbit Liver. J. Biol. Chem. 10 August 1980, Vol. 255, No. 15, pages 7161-7167, see entire document.	7 --- 1-6, 8-19
X,P --- Y,P	POTTER, P.M. et al. Isolation and Partial Characterization of a cDNA Encoding a Rabbit Liver Carboxylesterase That Activates the Prodrug Irinotecan (CPT-11). Cancer Research 15 June 1998, Vol. 58, pages 2646-2651, see entire document.	1, 2, 4-7 --- 3, 8-19
X,P ---- Y,P	POTTER, P.M. et al. Isolation and Characterization of a cDNA Encoding a Rabbit Carboxylesterase That Converts CPT-11 to SN-38. Proc. Amer. Assoc. Canc. Res. March 1998, Vol 39, page 421, see entire document.	1, 2, 4-7 --- 3, 8-19
X - Y	DANKS, M.K. et al. Overexpression of a Rabbit Liver Carboxylesterase Sensitizes Human Tumor Cells to CPT-11 Cancer Research 01 January 1998, Vol. 58, pages 20-22, see entire document.	1, 2, 4-7 --- 3, 8-19
X,P --- Y,P	POTTER, P.M. et al. Cellular Localization Domains of a Rabbit and a Human Carboxylesterase: Influence on Irinotecan (CPT-11) Metabolism by the Rabbit Enzyme. Cancer Research. 15 August 1998, Vol. 58, pages 3627-3632, see entire document.	1-7 --- 8-19
Y	WO 93/01281 A1 (MULLEN et al.) 21 January 1993, especially paes 4-6, 17, 21 and 22.	8-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03171

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03171

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 15/55, 15/63, 1/21, 5/10, 9/18; A61K 48/00; C12Q 1/68, 1/44

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-6, and 8-11, drawn to DNA, vectors and host cells encoding a carboxylesterase which is capable of metabolizing a chemotherapuetic prodrug to the active drug and methods of use thereof for sensitizing tumor cells to a chemotherapuetic prodrug.
Group II, claim 7, drawn to a carboxylesterase which is capable of metabolizing a chemotherapuetic prodrug to the active drug.
Group III, claims 12, 13, and 17, drawn to methods of inhibiting tumor growth.
Group IV, claims 14 and 15, drawn to methods of inhibiting tumor recurrence.
Group V, claim 16, drawn to methods of purging bone marrow cells.
Group VI, claims 18 and 19, drawn to methhods of assaying for drugs activated by carboxylesterase.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The DNA of Group I and protein of Group II do not share a corresponding special technical feature even though the DNA encodes the protein because the prior art clearly teaches a carboxylesterase which is capable of metabolizing a chemotherapuetic prodrug to the active drug (see for example the 1994 Sigma catalog, entries E2884 and E9686). Therefore the shared technical feature of these claims, i.e., the carboxylesterase, does not constitute a special technical feature as defined in PCT Rule 13.2 as it is not a feature which defines a contribution the claimed inventions make over the prior art. The methods of Groups III-VI do not share any technical feature with Group II and do not have unity of invention with Group I as Group I already includes a method of use of the carboxylesterase DNA which comprises unrelated steps to the methods of Groups III-VI and 37 CFR 1.475 does not provide for the inclusion of multiple methods of use within the main invention.